Outer Arm Dynein of Sperm Flagella and Cilia in the Animal Kingdom

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ABSTRACT

It has been established that the outer arm dynein molecule of flagella and cilia in Protozoa such as *Chlamydomonas* and *Tetrahymena* has a three-headed structure, whereas that of sperm flagella in sea urchins, fish and mammals exhibits a two-headed structure. All the latter animals belong to Deuterostomia. The outer arm dynein from sperm flagella of the oyster, a member of Protostomia, is also two-headed. Investigation has begun of the situation in other animals, especially in Cnidaria and Porifera. In *Chlamydomonas*, there is a mutant whose outer arm dynein lacks one head. Electron microscopy revealed that the outer arm of the wild type shows a pistol-like shape in the cross-section of the flagellar axoneme, whereas that of the mutant exhibits a hook- or fist-like shape. This suggests that we may predict the number of heads of outer arm dynein by observing the cross-sections of flagella or cilia. Examination of the cross-sections of sperm flagella and cilia in various animals, including mammals, tunicates, echinoderms, molluscs, annelids, arthropods, flatworms and sea anemones, indicated that their outer arms were hook- or fist-like, in contrast to the pistol-like shape in *Paramecium* and *Tetrahymena*. The former was true with choanocyte flagella of the sponge and metazoan cilia. The reduction in the number of heads of outer arm dynein molecule would have occurred during the evolution from Protozoa to Metazoa (and Mesozoa). Alternatively, the outer arm dynein in Protozoans was specialized to support their peculiar behaviour.

RÉSUMÉ

La dynéine du bras externe des flagelles des spermatozoïdes et des cils dans le Règne Animal

Il a été établi que la molécule de dynéine du bras externe des flagelles et des cils des Protozoaires, tels que *Chlamydomonas* et *Tetrahymena*, a une structure à trois têtes, alors que les flagelles des spermatozoïdes des Oursins, Poissons et Mammifères ont une structure à deux têtes. Tous les animaux précédemment cités appartiennent aux Deutérostomiens. La dynéine du bras externe de l'huître, un Protostomien, a aussi deux têtes. Les études sur d'autres animaux, spécialement des Cnidaires et des Porifères, ont commencé. Chez *Chlamydomonas*, il existe un mutant dont la dynéine du bras externe est dépourvue d'une des têtes. La microscopie électronique montre que le bras externe de type sauvage a une forme en pistolet sur les coupes transversales de l'axonème du flagelle, alors que celui du mutant a une forme en crochet ou en poing. Ceci suggère que nous pouvons prédire le nombre de têtes de la dynéine du bras externe en observant des coupes transversales de flagelles ou de cils. L'observation de coupes transversales de flagelles de spermatozoïdes et de cils d'animaux divers, tels que des Mammifères, Tuniciers, Échinodermes, Mollusques, Annélides, Arthropodes, Plathelminthes et Anémones de Mer, montre que leur bras externe est en forme de crochet ou de poing, en opposition avec la forme en pistolet de *Paramecium* et *Tetrahymena*. La même situation a été rencontrée pour les flagelles des choanocytes des Spongiaires et les cils des Métazoaires. La réduction du nombre de têtes de la molécule de dynéine du bras externe a pu intervenir lors de l'évolution depuis les Protozoaires vers les Métazoaires (et Mésozoaires). Une alternative serait d'interpréter la dynéine du bras externe des Protozoaires comme spécialisée pour permettre leur comportement particulier.

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Dynein was first thought to be a globular molecule with molecular mass of 300 000-600 000, representing either a single outer arm or an inner arm in the axoneme of flagella and cilia [7, 16]. The outer arm dynein has three heads connected by stalks to a common base in flagella or cilia of Protozoa such as *Chlamydomonas* [8, 26], *Tetrahymena* [11, 28] and *Paramecium* [13], as revealed by either negatively by stained or quick-freeze deep-etch images under the electron microscope. Biochemical analyses also indicated that there are three distinct heavy chains with molecular masses of 400 000 - 500 000, which constitute the corresponding heads, as well as a few intermediate chains and a few light chains. On the other hand, outer arm dynein obtained from sperm flagella of sea urchin [24], rainbow trout [6] and bull [14] has two heads and consists of the corresponding two heavy chains. Recent studies indicate that inner arm dynein is different from outer arm dynein not only in molecular composition [19] but also in function [4, 5]. A couple of different inner arm dynein molecules are present in a single flagellum and cilium, and they appear to be two-headed even in the case where outer arm dynein is three-headed [21, 25]. Phylogenetically, all the above-mentioned Metazoa belong to Deuterostomia.

We examined the outer arm dynein of sperm flagella in the oyster as a representative of Protostomia [30]. The purified dynein contained two heavy chains, and its negative stained image was two-headed under the electron microscope. However, the question arose of whether the two-headed dynein obtained is really an intact molecule or the product of a three-headed molecule as 18S dynein in *Chlamydomonas* is [33], although the same extraction procedure was used as that applied for outer arm dynein from sea urchin sperm flagella. It is desirable to check this point by some other means. Furthermore, from a phylogenetic point of view, it is interesting to know whether the outer arm dynein of flagella or cilia in other animals, especially Cnidaria, Porifera, etc., is two-headed or three-headed.

TABLE 1. — List of species examined. (s): sperm, (f): flagella, (c): cilia.

Protozoa	*Chlamydomonas reinhardtii* (f)
	Tetrahymena pyriformis (c)
	Paramecium caudatum (c)
Porifera	*Halichondria japonica* (f)
Cnidaria	*Anthopleura midori* (s)
Platyhelminthes	*Planoceros reticulata* (s)
Annelida	*Neanthes diversicolor* (s)
Mollusca	*Crassostrea gigas* (s, c)
	Mytilus edulis (s)
	Meretrix lusoria (c)
	Tapes japonica (c)
Arthropoda	*Balanus uliginosus* (s)
	Callophrys ferrea (s) and other butterflies (s)
Echinodermata	*Hemicentrotus pulcherrimus* (s)
	Pseudocentrotus depressus (s)
	Anthocidaris crassispina (s)
Protochordata	*Ascidia sydneiensis semea* (s)
Vertebrata	*Mus musculus* (s, c)

In *Chlamydomonas*, there is a flagellar mutant (oda-11) missing the α- heavy chain of outer arm dynein but retaining the β- and γ-chains [22]. In other words, the outer arm dynein molecule in this mutant is two-headed. Examination of the cross-sections of axonemes under the electron microscope indicates that the image of the outer arm in wild-type *Chlamydomonas* is pistol-like

(see Fig. 1a), whereas that in the mutant is hook- or fist-like (see Fig. 1b). This fact should provide us a useful tool for predicting the number of heads of outer arm dynein molecules *in situ*. In fact, the image of the outer arm, consisting of two-headed dynein, in the cross-section electron micrograph of the flagellar axoneme of sea urchin sperm resembles that of the flagellar axoneme of the mutant *Chlamydomonas* (see Fig. 1c). Since the starting materials are often insufficient in amount for biochemical analysis of a purified dynein molecule, such an examination of the image of the outer arm on the cross-sections of flagella or cilia seem to be effective in comparatively analyzing the number of heads of the outer arm dynein molecule in different animal species. In the present study, the outer arms of sperm flagella or cilia were examined in the species listed in Table 1. In the case of Porifera, choanocyte flagella were used as material instead of sperm flagella.

MATERIALS AND METHODS

Flagellar axonemes of the wild type and oda 11 mutant of *Chlamydomonas reinhardtii* isolated by the method of WITMAN [32] and ciliary axonemes of *Tetrahymena pyriformis* isolated by the method of TANAKA & MIKI-NOUMURA [27] were gifts from Dr. R. KAMIYA and Dr. T. MIKI-NOUMURA, respectively. Sperm flagella and axonemes of the sea urchins *Hemicentrotus pulcherrimus*, *Psudocentrotus depressus* and *Anthocidaris crassispina*, of the molluscs *Crassostrea gigas* and *Mytilus edulis*, and of the sea anemone *Anthopleura midori*, were prepared as previously described [10]. Spermatozoa of the mouse *Mus musculus*, of the tunicate *Ascidia sydneiensis semea*, of the cirripede *Balanus uliginosus*, of the annelid *Neanthes diversicolor*, and of the flatworm *Planoceros reticulata* were directly treated with a demembranation solution consisting of 0.1-0.4 % Triton X-100, 0.15 M KCl, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT and 20 mM Tris-HCl, pH 8.0. Intact *Paramecium caudatum*, a piece of mouse trachea and pieces of the gill of bivalves, *Crassostrea gigas*, *Meretrix lusoria* and *Tapes japonica*, were subjected to the demembranation solution. The sponge *Halichondria japonica* was minced with scissors, filtered through a mesh to remove spicules and then treated with the demembranation solution.

Demembranated axonemes of flagella and cilia (intact flagella in the cases of insect sperm) were fixed with a mixture of 2.5 % glutaraldehyde and 1 % tannic acid in 0.2 M sodium cacodylate, followed by either post-fixation with 1 % OsO_4 or block-staining with uranyl acetate. Specimens were dehydrated and embedded in Quetol 812 (Epon 812 in the case of *Chlamydomonas* flagella). Thin sections were made with a Sorvall ultra-microtome MT-2 and observed under a JEOL 1200A electron microscope. To examine the shape of the outer arms more thoroughly, images of the axonemes on cross-section electron micrographs were superimposed and averaged using an IBAS image analyzer.

RESULTS

Protozoan flagella and cilia

As mentioned in the Introduction, the outer arm of the wild-type *Chlamydomonas* flagella had a pistol-like shape on the cross-section electron micrograph (Fig. 1a). The image corresponds to a three-headed dynein molecule. On the other hand, that of the mutant (oda 11) flagella, in which the α-heavy chain of outer arm dynein molecule is missing, showed a hook- or fist-like image (Fig. 1b). Comparison of the averaged image of the outer arms between the wild type and the mutant clearly indicated that the distal portion of the outer arm is lost in the mutant flagella, as described by SAKAKIBARA *et al.* [22]. One head corresponding to the α-heavy chain must reside in this domain of the outer arm. As is shown in Fig. 2a, the image of the outer arm on the cross-section of *Tetrahymena* cilia was quite similar to that in the wild *Chlamydomonas* flagella. The same was true of *Paramecium* cilia (Fig. 2b). The results are consistent with the fact that isolated outer arm dynein molecules in these organisms are three-headed.

Metazoan flagella

Figure 1c shows the cross-section of the sea urchin sperm flagellum (*Hemicentrotus*) and the averaged image of the outer arm. It is clear that the image is hook- or fist-like, as in the case of the outer arm in the mutant *Chlamydomonas* flagellum. Figures 2c and 2e show the cross-sections of sperm flagella in the mouse and the oyster *Crassostrea*, whose outer arm dynein must be two-headed. In both cases the image of the outer arm was similar to that in the mutant *Chlamydomonas*. Thus the two-headed structure of outer arm dynein must be reflected in an image of the outer arm. The outer arm of sperm flagella in the tunicate *Ascidia*, a member of Deuterostomia as mammal and sea urchin, was also the two-headed type (Fig. 2d).

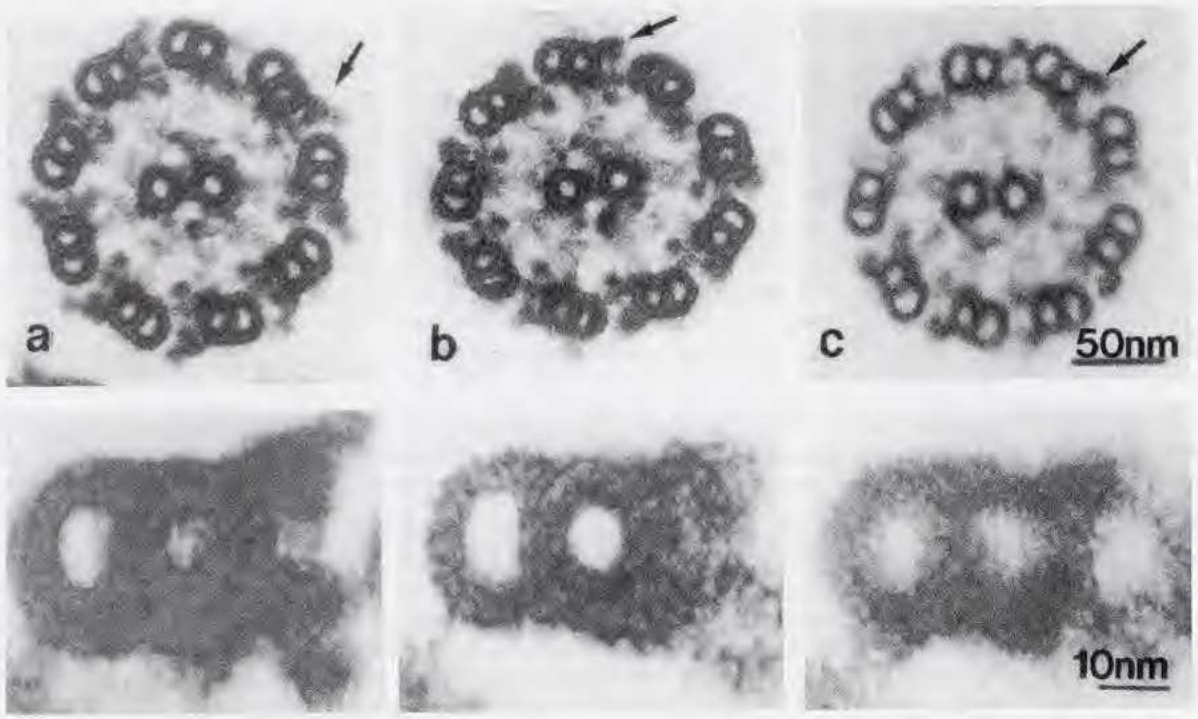

FIG. 1. — Cross-sections of axonemes with averaged outer doublet images. **a:** Flagellum of wild-type *Chlamydomonas*; **b:** Flagellum of a mutant (oda 11) *Chlamydomonas*; **c:** Sperm flagellum of the sea urchin *Hemicentrotus pulcherrimus*.

The results obtained with sperm flagella of other members of Protostomia are shown in Fig. 2g, h, i and j. They were the annelid *Neanthes*, the insect *Callophrys*, the cirripede *Balanus* and the flatworm *Planoceros*. The images of the outer arms in these species were also hook- or fist-like, indicating the two-headed molecule of outer arm dynein. Although not shown here, sperm flagella of many butterflies other than *Callophrys* were examined with the same result. In arthropods such as insects and cirripedes, the morphology of the outer arm was somewhat different from that in other animals, taking rather an axe-like shape (see also [1]). However, the image corresponding to the distal domain of the protozoan outer arm could not be found. It should be noted that the axoneme of flatworm sperm is a 9+"1" type.

Cnidaria is likely to be the phylogenetic ancestor of both Protostomia and Deuterostomia. A cross-section of sperm flagellum of the sea anemone, *Anthopleura*, is shown in Fig. 2k. The shape of the outer arm in this species was not different from that of sperm flagella in other metazoan animals. A preliminary biochemical analysis of purified outer arm dynein preparation in the sea anemone indicated the presence of two heavy chains on SDS polyacrylamide gel (data not shown). Furthermore, the outer arm of choanocyte flagella in the sponge *Halichondria* was not of the protozoan type (Fig. 2l).

Metazoan cilia

Since cilia were examined in *Tetrahymena* and *Paramecium*, and furthermore *Chlamydomonas* flagella beat like metazoan cilia in forward movement, metazoan cilia may possess outer arm dynein somewhat different from that of metazoan flagella. As can be seen from

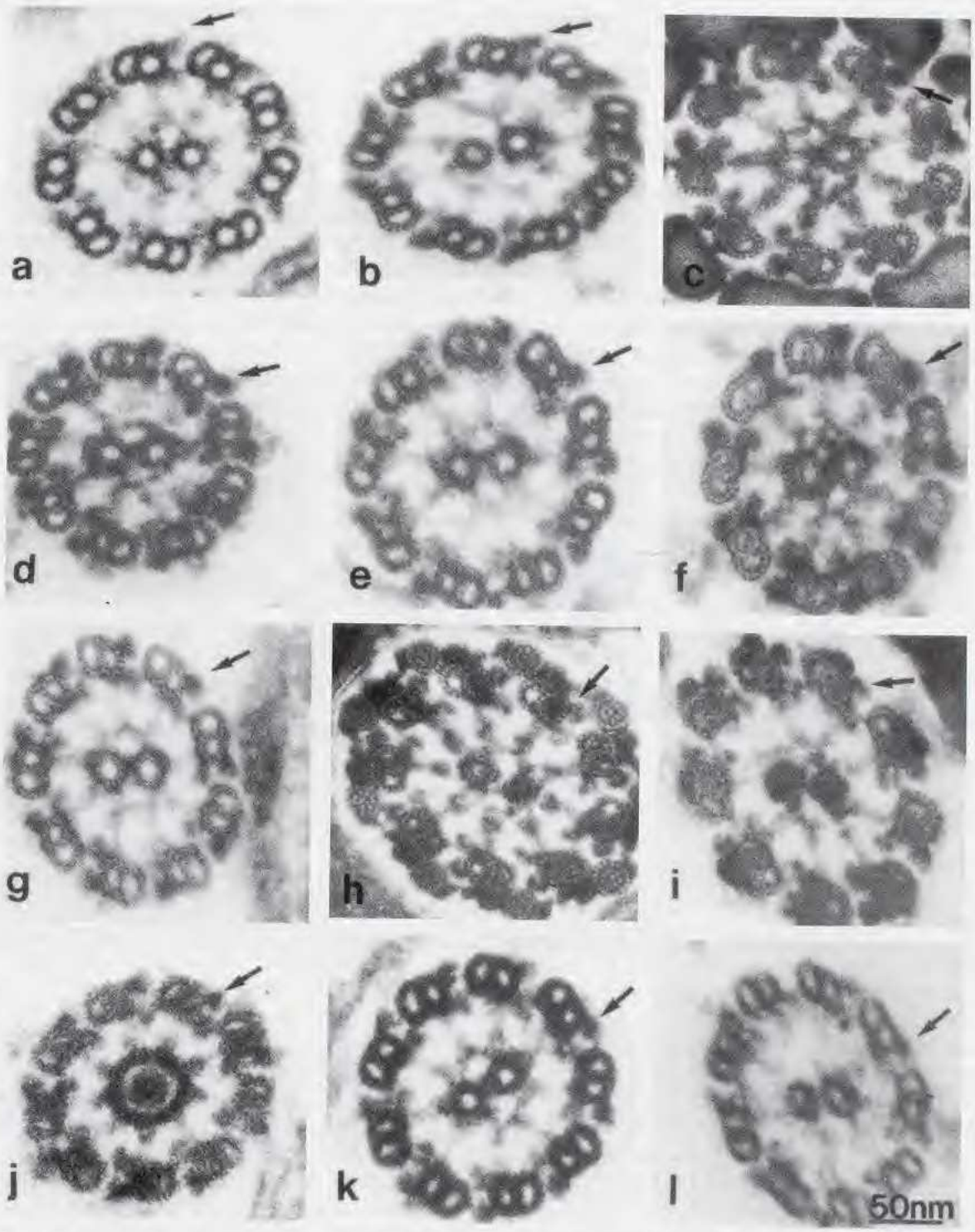

FIG. 2. — Cross-sections of flagellar and ciliary axonemes in various animals. **a:** Cilium of *Tetrahymena pyriformis*; **b:** Cilium of *Paramecium caudatum*; **c:** Sperm flagellum of the mouse *Mus musculus*; **d:** Sperm flagellum of the tunicate *Ascidia sydneiensis semea*; **e:** Sperm flagellum of the oyster *Crassostrea gigas*; **f:** Gill cilium of the oyster; **g:** Sperm flagellum of the annelid *Neanthes diversicolor*; **h:** Sperm flagellum of the butterfly *Callophrys ferrea*; **i:** Sperm flagellum of the cirripede *Balanus uliginosus*; **j:** Sperm flagellum of the flatworm *Planoceros reticulata*; **k:** Sperm flagellum of the sea anemone *Anthopleura midori*; **l:** Choanocyte flagellum of the sponge *Halichondria japonica*. Arrows indicate the outer arms.

Fig. 2f, the outer arm of gill cilia in the oyster shows the same image as that of sperm flagella of the same species. The results were the same with gill cilia of other bivalves, *Meretrix* and *Tapes*, and with tracheal cilia of the mouse.

DISCUSSION

Morphology of the outer arm

The presence of several dynein species with somewhat different functions has been disclosed within a single flagellum or cilium [20, 29]. According to a recent review by BROKAW [4], the main function of outer arm dynein in flagella and cilia appears to be generation of power to overcome viscous resistance, in contrast to the functions of inner arm dyneins, which appear to be bend initiation and maintenance of the angle of propagating bend. Outer arm dynein molecules so far purified from protozoan flagella and cilia possess three heads corresponding to three different heavy chains, α, β and γ, obtained by SDS-PAGE [8, 11, 13, 26, 28]. On the other hand, purified outer arm dynein molecules of sperm flagella in Metazoa such as mammals, fish, sea urchins and molluscs, consist of only two heads and two heavy chains, α and β [10, 13, 24, 30].

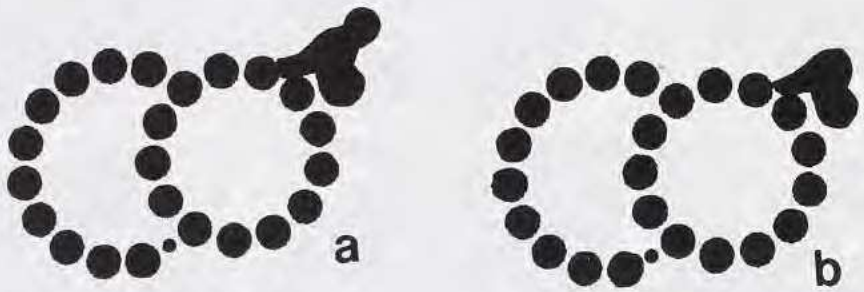

FIG. 3. — Schematic cross-section of an outer doublet microtubule with the outer arm. **a:** protozoan axoneme; **b:** metazoan axoneme.

Recent studies on *Chlamydomonas* flagellar mutants by KAMIYA and his colleagues [22, 23] clearly showed that one head corresponding to the α-heavy chain is situated at the distal domain of the outer arm, which looks like a pistol on the axoneme cross-section, the other two heads compose the body domain of the arm, one head corresponding to the γ-heavy chain localized at the innermost domain. Only the body domain of the outer arm is found in a flagellar mutant, oda 11, of *Chlamydomonas*, which lacks the α-heavy chain, resulting in a hook-like image of the outer arm on the cross-section electron micrograph. The present results indicate that in the metazoan sperm flagella from which two-headed outer arm dynein molecules have been isolated, the outer arms show the hook-like image representing the body domain of the outer arm in protozoan axonemes. In other words, α- and β-heavy chains of metazoan outer arm dynein would correspond to β- and γ-heavy chains of protozoan outer arm dynein. This fact also proves that the outer arm dynein molecule of oyster sperm flagella is certainly two-headed *in situ*, as indicated by biochemical analysis and the negative stained image of isolated dynein [30].

Most schematic cross-sections of flagellar and ciliary axonemes are based on electron micrographs obtained with either *Tetrahymena* cilia [2] or molluscan gill cilia [31], and have been accepted as common to all kinds of flagella and cilia [e.g: 3, 15]. Little attention has been paid to the image of the outer arm, in spite of the above-mentioned difference in the number of heads of

outer arm dynein. In the most recent diagram depicting the *Chlamydomonas* axoneme, the outer arm is drawn as it is in Fig. 1a in this paper ([12]; see also computer-generated cross-section of the axoneme in [9]). Therefore, a different image should be drawn for the metazoan axoneme. On the basis of the present findings, we propose here an interpretative diagram of the outer arms in protozoan and metazoan axonemes as represented in Fig. 3.

Phylogenetic aspects

Phylogenetically, sperm flagella of metazoan animals belonging to Protostomia and Deuterostomia exhibit the same morphology of the outer arm, which incorporates the two-headed structure of outer arm dynein. The outer arm of flatworm sperm flagella which have a 9+"1" axoneme and show three-dimensional movement is also not exceptional. No difference is observed with ciliary axonemes of these metazoan species, and outer arm dynein from embryonic cilia of the sea urchin has been reported to contain only two heavy chains [18]. Thus motility pattern does not seem to reflect on the structure of outer arm dynein among Metazoa.

An axe-like image of the outer arm in the cross-section electron micrographs of arthropod sperm flagella (Fig. 2h, i) would be caused by a slight shift of the two heads inward as compared with the arrangement of these heads in other metazoan axonemes. Down the phylogenetic tree to Cnidaria and Porifera, the common ancestors of both Protostomia and Deuterostomia, outer arm dynein molecules are considered to be two-headed. Furthermore, the image of the outer arm in the cross-section of the ciliary axoneme of Mesozoa, *Dicyema misakiensis*, is also hook- or fist-like, suggesting the two-headed structure of constituent dynein (Y. YAMAKAWA & M. OKUNO, personal communication). Although more thorough examination would be needed, so far only protozoan flagella and cilia have outer arm dynein with a three-headed structure.

Hence the reduction in the number of heads of outer arm dynein molecule would have occurred during evolution from Protozoa to Mesozoa and Metazoa, as an adaptation to simplify and make more efficient the motile machinery of the latter's flagella and cilia. Alternatively, the outer arm dynein in protozoan flagella and cilia was rather specialized. The addition of an extra head would facilitate complex movements such as the cilia-like motion in forward swimming and flagella-like motion in backward swimming of *Chlamydomonas* as each of the heads of outer arm dynein appears to exhibit a somewhat different function from the others [17].

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